HHS Public Access

Author manuscript

Microvasc Res. Author manuscript; available in PMC 2020 July 01.

Published in final edited form as:

Microvasc Res. 2019 July ; 124: 67–75. doi:10.1016/j.mvr.2019.03.006.

DLL4 and Jagged1 are Angiogenic Targets of Orphan Nuclear Receptor TR3/Nur77

Jin Peng[1,2,*], **Shengqiang Zhao**[1,3,*], **Yan Li**[1,3], **Gengming Niu**[1,4], **Chen Chen**[1,5], **Taiyang Ye**[1,6], **Dezheng Zhao**[1], and **Huiyan Zeng**[1,$]

[1]Center for Vascular Biology Research and Division of Gastroenterology, Departments of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston MA 02215, USA;

[2]Radiotherapy and Medical Oncology Department, Zhongnan Hospital, Wuhan University, Wuhan, P. R. China.

[3]Department of Gastroenterology, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, 250021, P. R. China.

[4]Department of General Surgery, Huashan Hospital, Fudan University, Shanghai, 200040, P.R. China;

[5]Department of Surgery of Breast and Thyroid, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, 430022, China;

[6]Department of Obstetrics & Gynecology, Renji Hospital, Shanghai Jiao Tong University, School of Medicine, Shanghai, 200127, P. R. China.

Abstract

Pathological angiogenesis is a hallmark of many diseases. Previously, we reported that orphan nuclear receptor TR3/Nur77 was a critical mediator of angiogenesis to regulate tumor growth and skin wound healing via regulating the expression of the junctional proteins and integrins. However, the molecular mechanism, by which TR3/Nur77 regulates angiogenesis is not completely understood. Here, we were the first to find that TR3/Nur77, via its various amino acid fragments, regulated the expression of DLL4 and Jagged 1 in cultured endothelial cells. DLL4 and Jagged1 mediated TR3/Nur77-induced angiogenic responses and signaling molecules, but not the expression of integrins. Instead, integrins regulated the expressions of DLL4 and Jagged1 induced by TR3/Nur77. Further, DLL4, Jagged1 and integrins α1, α2, β3 and β5 were regulated by TR3/Nur77 in animal sepsis models of lipopolysaccharide (LPS)-induced endotoxemia, and cecal ligation and puncture (CLP), in which, TR3/Nur77 expression was significantly and tranciently increased. Mouse survival rates were greatly increased in Nur77 knockout mice bearing both CLP

[$]**Address for Correspondence:** Huiyan Zeng, Ph.D., Division of Gastroenterology, Department of Medicine, Beth Israel Deaconess Medical Center, and Harvard Medical School, 99 Brookline Ave. RN 270F, Boston, MA 02215, Telephone: 617-667-2329, Fax: 617-667-3591, hzeng@bidmc.harvard.edu.

[*]Equal first authors,

and LPS models. The results elucidated a novel axis of VEGF / histamine → TR3/Nur77 → integrins → DLL4 / Jagged1 in angiogenesis, and demonstrated that TR3/Nur77 was an excellent target for sepsis. These studies supported our previous findings that TR3/Nur77 was an excellent therapeutic target, and further our understanding of the molecular mechanism, by which TR3/Nur77 regulated angiogenesis.

Keywords

TR3/Nur77; angiogenesis; sepsis; DLL4; Jagged1; integrin

Introduction

Pathological angiogenesis is a hallmark of many diseases including cancer, inflammation, wound healing, and ischemic heart diseases. Neutralizing antibodies against vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor (VEGFR) kinase/multiple kinase inhibitors have been successfully developed and widely used in the clinic (reviewed in (1)). However, in addition to their side effects (2), VEGF-targeted therapies face the problems of insufficient efficacy (3–12), resistance and intrinsic refractoriness (10, 13, 14). Therefore, it is desirable to identify additional angiogenic targets. Our studies demonstrated that TR3/Nur77 (human: TR3, mouse: Nur77, rat: NGFI-B) was one of such promising targets (15–17).

TR3/Nur77 is a member of the nuclear receptor IV subfamily of the transcription factors, without identified physiological ligand (18), although several agonists, including cytosporone B and a series of methylene-substituted diindolymethanes, were identified (19, 20). The nuclear receptor IV subfamily members play redundant roles in TCR-mediated apoptosis (21) and brown fat thermogenesis (22, 23). However, they play different roles in development (reviewed in (24)). TR3/Nur77 also plays important roles in cancer cell biology, inflammation, metabolic diseases, stress and addiction (reviewed in (25–28)).

Prior to our studies (16), little was known about the role of TR3/Nur77 in angiogenesis. Our studies demonstrated that TR3/Nur77 was a critical mediator of angiogenesis. TR3/Nur77 was highly and transiently up-regulated in cultured endothelial cells (EC) and during angiogenesis *in vivo*. TR3/Nur77 was induced by the angiogenic factors having microvessel permeable activity, including VEGF, histamine and serotonin, but not by the angiogenic factors without microvessel permeable activity, including basic fibroblast growth factor (bFGF), placental growth factor (PlGF) and platelet-derived growth factor PDGF (15–17), and in postnatal angiogenesis, such as tumor angiogenesis and skin wound healing (16, 29). In the loss of function assays, the knockdown of TR3 expression by its antisense DNA or shRNA inhibited endothelial cell proliferation, migration and tube formation induced by VEGF, histamine and serotonin *in vitro* (15–17). Tumor growth, angiogenesis and microvessel permeability induced by VEGF, histamine or serotonin were almost completely inhibited in Nur77 knockout mice (15–17). Paradoxically, however, Nur77 null mice are viable, fertile, appear to develop a normal adult vasculature and have no defect on normal skin wound healing (21, 29). In the gain of function assays, the overexpression of TR3/

Nur77 protein was sufficient to induce endothelial cell proliferation, migration and tube formation *in vitro*. Angiogenesis, microvessel permeability and normal skin wound healing were greatly induced/improved in our transgenic EC-Nur77-S mice, in which the full length Nur77 was inducibly and specifically expressed in mouse endothelium (15–17). The transgenic EC-Nur77-S mice were healthy after Nur77 had been induced for three months (29). Our studies demonstrated that TR3/Nur77 was an excellent target for pro-angiogenesis and anti-angiogenesis therapies. Our studies further demonstrated that TR3/Nur77 regulated angiogenesis in the early stage by regulating the expression of eNOS, protein components in VE-cadherin associated adherent junctions, and integrins (15–17, 29, 30).

However, the molecular mechanism, by which TR3/Nur77 regulated angiogenesis was not completely understood. In this study, we demonstrated that DLL4 and Jagged1 were norvel downstream targets of TR3/Nur77 and integrins, and that TR3/Nur77 regulated the development of sepsis. DLL4, Jagged1 and integrins α1, α2, β3 and β5 were regulated by TR3/Nur77 in animal sepsis models. This study supported our previous findings that TR3/Nur77 was an excellent therapeutic target, and furthered our understanding of the molecular mechanism by which TR3/Nur77 regulated angiogenesis.

Materials and Methods

Materials

The recombinant human VEGF was purchased from R&D Systems (Minneapolis, MN). Histamine and Flag antibody (Cat. No. F-3165) were purchased from Sigma (Sigma-Aldrich Co. LLC, St. Louis, MO). The antibodies against pAkt-S473 (Cat. No. 9271), Akt (Cat. No. 9272), phospho-p42/p44 MAPK (Cat. No. 9106S) and p42/p44 MAPK (Cat. No. 9211) were from Cell Signaling Technology, Inc. (Danvers, MA, USA). The antibodies against TR3/Nur77 (Cat. No. sc-5569), integrin α2 (Cat. No. SC-6586), integrin β3 (Cat. No. SC-14009) and integrin β5 (Cat. No. SC-14010) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The integrin α1 antibody (MAB1973) was purchased from EMD Millipore (Billerica, MA). Endothelial cell basal medium (EBM), EGM-MV BulletKit, Trypsin/EDTA, and trypsin neutralization solution were obtained from Lonza (Allendale, NJ). Vitrogen 100 was purchased from Collagen Biomaterials (Palo Alto, CA, USA).

Cell Culture

Primary human umbilical vein endothelial cells (HUVECs) purchased from Lonza (Allendale, NJ) were grown on the plates coated with 30 μg/ml vitrogen in EBM supplemented with EGM-MV BulletKit. The HUVECs of passages 5 were used for all of the experiments.

Adenoviruses expressing shRNAs and cDNAs of DLL4 and Jagged1

The shRNA oligonucleotides were ordered from Integrated DNA Technologies (Coralville, Iowa), then annealed and cloned to a pENTR1A-stuffer vector that was modified from the lentiviral vector pLKO.1 (Addgene, Boston, MA) with a pU6 promoter and a PURO element (30), via the restriction enzymes AgeI and EcoRI. The pENTR1A-shRNA plasmids were

sequenced corrected, used as the entry clones and transferred to the pAd/PL-DEST vector following the instruction provided by Invitrogen (Carlsbad, CA).

The DLL4 cDNA were obtained by RT-PCR with the RNA isolated from HUVECs using the primer pairs of DLL4 cDNA F1.XhoI and DLL4 cDNA R2082.BamHI. The PCR products were cloned to pCMBP vector (30) via blunted N-terminus and the restriction enzyme BamHI, and sequenced corrected.

To clone the Jagged1 cDNA, the N-terminus and the C-terminus of the Jagged1 cDNA were obtained by RT-PCR with the RNA isolated from HUVECs using the primer pairs of 1) Jagged-1 F1 and Jagged1 R2096, and 2) Jagged1 cDNA F1931 and Jagged1 cDNA R3657.BAM HI. The two PCR products were digested with the restriction enzymes XhoI + NruI, and NruI + BamHI, respectively, and then ligated into the pCR2.1-TOPO vector that was digested with the restriction enzymes XhoI and BamHI, to obtain the plasmid pCR2.1-TOPO-Jagged1. The coding region was sequenced correct. The Jagged1 cDNA was digested from the pCR2.1-TOPOJagged1 with the restriction enzymes XhoI and BamHI, and subcloned into the retroviral expression vector pCMBP (31) to obtain the pCMBP-Jagged1 cDNA plasmid.

All of the sequences were listed in the supplemental data.

Proliferation assay

Twenty-four hours after seeded in 96-well plates (2×10^3 cells / well), the HUVECs were transduced with the viruses as indicated. Forty-eight hours after transduction, the cells were serum-starved with 0.1% fetal bovine serum (FBS) in EBM for 48 hours. The Cell Counting Kit-8 reagent (CK04–11, Dojindo) was added to each well. The plates were incubated for 3 hours before the absorbance at 450 nm was measured using a microplate reader.

Monolayer migration assay

The HUVEC monolayer migration assay was carried out as described previously (15). Briefly, HUVECs (6×10^4 cells / well) were seeded in 6-well plates. Twenty-four hours later, the cells were transduced with the viruses as indicated. Forty-eight hours later, the cells were serum-starved with 1% FBS in EBM for 24 h. Scratch wound was generated with a 200 μl pipette tip and photographed immediately as 0 h, and at 16 h, respectively. The cells migrated to the wound area were counted. The results were expressed as mean ±SD of 6 views.

Cell adhesion assay

HUVECs (1.5×10^5 cells / plate) were seeded in 60 mm plates. Twenty-four hours later, the cells were transduced with the viruses as indicated. Forty-eight hours later, the cells were serum-starved with 1% FBS in EBM for 48 hours, or serum-starved for 24 hours and stimulated with VEGF for 24 hours. Then, the cells were harvested with 0.25% trypsin, centrifuged at 1200 rpm for 3 min, washed with 10 ml of Phosphate Buffered Saline (PBS) for 3 times, and resuspended in 800 μl of adhesion buffer that contains RPMI1640 medium, 1 mM $MgCl_2$ and 0.5% BSA. The cells (1×10^5 cells / well) were seeded on 48-well plates.

After incubated for 30 min, the cells were washed 3 times with warm PBS, stained with 0.1% crystal violet (100 μl / well) for 10 min, washed with warm PBS 3 times carefully, and incubated with 100 μl of 10% acetic acid at room temperature. The optical density (OD) value was measured at 600 nm using a microplate reader.

Quantitative real-time RT-PCR

RNA was isolated from cells or septic tissues, reverse-transcribed and subjected to real-time RT-PCR. The sequences for real-time RT-PCR primers were listed in the supplemental data. GAPDH served as an internal control.

Septic models of endotoxemia and CLP

LPS injections and CLP models were performed in male and female 8–10-week-old mice. For the endotoxemia model, mice were injected i.p. with 13 mg / kg LPS or the same amount of HBSS. CLP was performed as previous report (32). Briefly, mice were anesthetized with isoflurane on the water-circulating heat pad. After shaving the abdomen, a midline incision of 2 cm was made on the mid-lower part of the abdomen with a sterilized #11 blade scalpel to open the peritoneum using blunt dissection under aseptic conditions to expose the cecum and adjoining intestine. The cecum together with a part of the ascending colon was taken out. The end of the cecum was filled with feces. Then the cecum was ligated at 3/5 mm from the end with 6–0 vicryl suture. The ligated part was punctured with a 21 G needle. Some of the feces were driven out to make a 2~3 mm diameter pellet. Finally, The cecum was gently put back to the left-lower part of the abdomen with forceps and the peritoneum was closed with a running 6–0 absorbable vicryl suture, and the skin was interrupted with non-absorbable suture. Mice were injected with prewarmed sterilized normal saline (37 °C, 5 ml for 100 g body weight) subcutaneously at right-lower abdomen at the time of closure and a dose of 0.05 mg / kg buprenorphine was injected subcutaneously every 12 h for postoperative analgesia until sacrifice.

Survival studies

Survival studies were performed using endotoxemia and CLP models. Mice were monitored every 4 hours after LPS injection or CLP preformed. Death events were recorded. The data were analyzed by Prism software and the survival curves were created.

Animal welfare

All animal experiments were performed in compliance with the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee.

Statistical analysis

Results were presented as mean ± SD. Student’s t-test and ANOVA with *post-hoc* test for multiple comparisons were employed to determine statistical significance. P values less than 0.05 were considered as statistically significant.

Results

TR3/Nur77 mediated the expression of DLL4 and Jagged1 regulated by VEGF and histamine.

Because TR3/Nur77 was a common downstream target of VEGF and histamine (15, 16), we studied whether histamine, similar to VEGF, regulated the expression of DLL4 and Jagged1. Serum-starved HUVECs were stimulated with or without VEGF and histamine for various time periods as indicated. Cellular extracts were subjected to immunoblotting with antibodies against DLL4, Jagged1 and β-actin as the protein equal loading control. VEGF and histamine up-regulated DLL4, but downregulated Jagged1 (Fig. 1A and B). We knocked down the expression of TR3/Nur77 to test whether TR3/Nur77 mediated the up-regulation of DLL4 and the down-regulation of Jagged1 induced by VEGF and histamine. HUVECs were transduced with or without adenoviruses expressing TR3 shRNAs, shTR3–176 and shTR3–297, or shGFP as a control for 48 hours. After serum starvation, cells were treated with and without VEGF for 24 hours. Both shTR3–176 and shTR3–297 decreased and increased the expression of DLL4 and Jagged1 in the presence and absence of VEGF treatment, respectively (Fig. 1C). Then, we studied whether overexpressing TR3 cDNA had any effects on the expression of DLL4 and Jagged1. HUVECs were transduced with the adenoviruses expressing Lac Z as a control and TR3/Nur77. The proteins were collected at various time points after viral transduction and subjected to immunoblotting with antibodies again TR3/Nur77, DLL4, Jagged1 and β-actin. TR3/Nur77 was expressed from 8 hours after viral transduction (Fig. 1D, top panel). Overexpression of TR3/Nur77 down-regulated DLL4 expression (Fig. 1D, 2nd panel). Jagged1 expression was, as expected, almost completely inhibited after TR3/Nur77 was expressed (Fig. 1D, 3rd panel). These data indicated that TR3/Nur77 regulated the expression of DLL4 and Jagged1.

DLL4 and Jagged1 regulated the angiogenic responses induced by TR3/Nur77.

To further study the function of DLL4 and Jagged1 in TR3/Nur77-induced angiogenesis, we used the loss-of-function assays. Because overexpression of TR3/Nur77 down-regulated DLL4 and Jagged1, the full-length cDNAs of DLL4 and Jagged1 were cloned and over-expressed to prevent their down-regulations. The HUVECs expressing Flag-TR3 were transduced with Lac Z as a control, DLL4 and Jagged1 cDNAs. The cellular extracts were subjected to immunoblotting with the antibodies against Flag to confirm the equal expression of Flag-TR3, and β-actin as protein equal loading control. Antibodies against DLL4, Jagged1 indicated the overexpression of proteins (Fig. 2. A–I). The HUVECs expressing Flag-TR3 were transduced with the adenoviruses expressing these DLL4 and Jagged1 cDNAs and subjected to cell proliferation, migration and adhesion assays as described in detail in the Section of Materials and Methods. The TR3/Nur77-induced cell proliferation was significantly inhibited by DLL4 overexpression, but not affected by Jagged1 overexpression (Fig. 2. B–I). The overexpression of DLL4 and Jagged1 significantly inhibited cell migration and adhesion (Fig.2. C-I and D-I, and supplemental data Fig.S–A). In order to further study the function of DLL4 in angiogenesis, we generated DLL4 shRNAs, shDLL4–2437 and shDLL4–796. The HUVECs expressing Flag-TR3 were transduced with shGFP as control, shDLL4–2437 and shDLL4–796. The cellular extracts were subjected to immunoblotting with the antibodies against Flag to confirm the equal

expression of Flag-TR3, and β-actin as the protein equal loading control. The antibody against DLL4 indicated the knockdown of DLL4 (Fig. 2. A-II). The HUVECs expressing Flag-TR3 were transduced with the adenoviruses expressing shDLL4–2437 or shDLL4–796 and subjected to cell proliferation, migration and adhesion assays. Both shDLL4–2437 and shDLL4–796 significantly increased cell proliferation, migration and adhesion (Fig. 2. B-II, C-II and D-II, and supplemental Fig. S–B). These data clearly demonstrated that DLL4 and Jagg1 mediated the cell proliferation, migration and adhesion induced by TR3/Nur77.

Previously, we reported that TR3/Nur77 inhibited the expression and phosphorylation of Akt, but increased the phosphorylation of MAPK42/44 without affecting the total MAPK42/44 level (29). We tested whether DLL4 and Jagged1 influenced the effects of TR3/Nur77 on the expression of Akt and MAPK42/44, and their phosphorylation. HUVECs were transduced with the adenoviruses expressing TR3/Nur77, together with DLL4 and Jagged1 cDNAs. The cellular extracts were subjected to immunoblotting with the antibodies against phosphorylated AKT, AKT, phosphorylated MAPK42/44 and MAPK42/44. The expression of DLL4 and Jagged1 increased the phosphorylation of MAPK42/44, but did not affect the expression of MAPK42/44, and the phosphorylation and expression of AKT (Fig. 2E). These data indicated that DLL4 and Jagged1 differentially mediated the activation of signaling molecules induced by TR3/Nur77.

Integrins differentially regulated the down-regulation of DLL4 and Jagg1 induced by TR3/Nur77.

Most recently, we reported that the TR3/Nur77 highly increased the expression of integrins α1 and β5, decreased the expression of integrins α2 and β3, but had little effect on the expression of integrins αv, α3, α4, α5, α6, β1 and β7 in HUVEC (30). We would like to study whether DLL4 and Jagged1 regulated the expression of integrins induced by TR3/Nur77. HUVECs expressing Flag-TR3 were transduced with Lac Z as a control, Jagged1 and DLL4 cDNAs (Fig. 3A, panels I), shGFP as a control, shDLL4–2437 and shDLL4–796 (Fig. 3A, panels II). Cellular extracts were used for immunoblotting with antibodies against integrins α1, β5, α2 and β3. The expression of these integrins were not affected by Jagged1 and DLL4 overexpression, nor by DLL4 shRNAs (Fig. 3A, panels I and II). Then, we tested whether integrins regulated the down-regulation of DLL4 and Jagged1 induced by TR3/Nur77. HUVECs expressing Flag-TR3 were transduced with shGFP as a control, integrin α1 shRNAs (shITGα1–596 and shITGα1–1213) (Fig. 3B, panel I), shGFP as a control, integrin β5 shRNA (shITGβ5) (Fig. 3B, panel II), Lac Z as a control and integrin α2 cDNA (Fig. 3B, panel III), Lac Z as a control and integrin β3 cDNA (Fig. 3B, panel IV). Cellular extracts were used for immunoblotting with antibodies against DLL4 and Jagged1. shITGα1–596 and shITGα1–1213 increased the expression of DLL4, but did not affect the expression of Jagged1 (Fig. 3B, panel I). shITGβ5 increased the expression of DLL4 and Jagged1 (Fig. 3B, panel II). Integrins α2 and β3 cDNAs decreased the DLL4 expression, but increased Jagged1 expression (Fig. 3B, panels III and IV). These data showed that integrins regulated the expression of DLL4 and Jagged1, but DLL4 and Jagged1 did not regulate the expression of integrins in HUVECs expressing TR3/Nur77.

Various TR3/Nur77 domains were required for its regulation of WLL4 and Jagged1.

Previously, we reported that the transactivation domain and the DNA binding domain, but not the ligand-binding domain, of TR3/Nur77, were required for the function of TR3/Nur77 in angiogenesis ((15–17, 33) and Fig. 4A). We studied which TR3/Nur77 domain(s) played a role in its regulation of DLL4 and Jagged1. We found that both of the TR3ΔTAD and the TR3ΔDBD, in which the transactivation domain and the DNA-binding domain of TR3 are deleted, respectively, were unable to down-regulate the expression of DLL4 and Jagged1, but TR3ΔLBD (deletion mutant of ligand-binding domain) had the similar effects as TR3/Nur77 on regulating the DLL4 and Jagged1 expression (Fig. 4B). Our data indicated that the transactivation domain and the DNA binding domain, but not the ligand-binding domain, of TR3/Nur77 were required for its regulation of the DLL4 and Jagged1 expression. We also generated the serial deletions, TR3Δ1–20, TR3Δ21–40, TR3Δ41–60, TR3Δ61–80 and TR3Δ101–120, in which, the protein sequences of 1–20, 21–40, 41–60, 61–80 and 101–120 within the transactivation domain of TR3/Nur77 were deleted, respectively ((33) and Fig. 4C). HUVECs were transduced with the adenovirus expressing Flag-fused Lac Z as a control, TR3 cDNA, TR3Δ1–20, TR3Δ21–40, TR3Δ41–60, TR3Δ61–80 and TR3Δ101–120, respectively. The cellular extracts were subjected to immunoblotting analysis with an antibody against Flag to confirm the equal expression of the various mutants, and then with the antibodies against DLL4 and Jagged1. All of the deletion mutants were able to down-regulate the expression of DLL4, except TR3Δ41–60 (Fig. 4D, left panel). Jagged1 expression was, similar to TR3 cDNA, down-regulated by TR3Δ1–20 and TR3Δ21–40, but not down-regulated by deletion mutants TR3Δ41–60, TR3Δ61–80 and TR3Δ101–120 (Fig. 4D, left panel). Within the DNA binding domain, we generated the point mutants of TR3(S350A), TR3(S350D) and the deletion mutant TR3ΔGRR (33). The cellular extracts from HUVECs that were transduced with Flag-fused Lac Z as a control, TR3(S350A), TR3(S350D) and TR3ΔGRR were subjected to immunoblotting analysis with the antibodies against Flag, DLL4 and Jagged1. Similar to TR3 cDNA, TR3(S350A) down-regulated the expression of DLL4 and Jagged1. However, TR3(S350D) and TR3ΔGRR were unable to down-regulate the expression of DLL4 and Jagged1 (Fig. 4D, right panels). These data indicated that various amino acid fragments of TR3/Nur77 were required for its regulation of DLL4 and Jagged1.

TR3/Nur77 played a role in sepsis.

Next, we study whether TR3/Nur77 regulated the expression of DLL4, Jagged1 *in vivo*. Previously, we reported that the expression of TR3/Nur77 was required for the progression of tumor, but was not required for the normal skin wound healing (16, 17, 29). However, the overexpression of TR3/Nur77 in mouse endothelium did accelerate normal skin wound healing (16, 17, 29). We would like to study whether TR3/Nur77 played a role in sepsis. Mice were subjected to the CLP-induced sepsis or sham control. Twenty-four and forty-eight hours after surgery, RNA was isolated from mouse kidneys, and then subjected to Realtime PCR with the Nur77 specific primers. TR3/Nur77 expression was significantly increased about 5 fold at 24 hours after surgery (Fig. 5A). Then, we studied whether Nur77 regulated sepsis. The CLP-induced sepsis was performed on both males and females of Nur77 knockout mice and wild-type mice as a control. The mouse survival rate in Nur77−/− mice was about 90%, which was significantly different from that in wild-type mice, about

40% (Fig. 1B, a). The survival rates of Nur77−/− mice vs. wild-type mice were significantly different, no matter in male or female mice (Fig. 5B, b and c). We further studied the impact of Nur77 knockout on sepsis induced by the injection of LPS. However, the survival rate in Nur77−/− mice was not significantly different from that in wild-type mice (Fig. 5B, d). We analyzed the data for male and female mice, respectively, and found that the mouse survival rate in Nur77−/− mice bearing LPS-induced sepsis was significantly different from that in wild type control mice in males, but not in females (Fig. 5B, e and f). Our data clearly indicated that deletion of TR3/Nur77 increased survival rate in mice bearing sepsis induced by CLP and LPS.

The expression of DLL4, Jagged1 and integrins were regulated by TR3/Nur77 in sepsis.

We would like to study whether TR3/Nur77 regulated the expression of DLL4, Jagged 1, and integrins α1, β5, α2 and β3 in sepsis. Realtime RT-PCR with primers specific for DLL4, Jagged 1, and integrins α1, α2, β3 and β5 were carried out with the RNA that was isolated from kidneys, livers, spleens and lungs of male Nur77 knockout mice and their wild type control littermates 24 hours after CLP surgery. The expressions of integrins α1, α2, β3 and β5 were significantly decreased in kidneys, livers, lungs and spleens, except that integrin β3 and β5 were increased in livers and spleens, respectively, in Nur77−/− mice as compared to control wild type mice (Fig. 6). DLL4 was significantly decreased in kidneys, livers and lungs, but increased in spleens (Fig. 6). Jagged1 was significantly decreased in kidneys, lungs and spleens, but increased in livers (Fig. 6). Our data indicated that TR3/Nur77 regulated the expression of DLL4, Jagged 1, and integrins α1, β5, α2 and β3 in sepsis.

Discussion

Although there is no obvious phenotype in Nur77−/− mice, TR3/Nur77 plays important roles in carcinogenesis, apoptosis, brown fat thermogenesis, inflammation, metabolism diseases, stress and addiction (reviewed in (25–28)) as well as pathological angiogenesis (15–17), by regulating survival/growth and apoptosis of various types of cells (review in (34)). We also found that TR3/Nur77 regulated angiogenesis in the early stage by mediating microvessel permeability via regulating the expression of VE-cadherin, β-catenin, γ-catenin, P120, and claudin-5 (17), and by regulating the expression of integrins {Ye, 2018 #4121}. Here, we found that TR3/Nur77 regulated the expression of DLL4 and Jagged1 in culture endothelial cells, and that DLL and Jagged1 played important roles in the angiogenic responses induced by TR3/Nur77. Most recently, we reported that integrins α1, α2, β3, and β5 were down-stream targets of TR3/Nur77 in angiogenesis. Here, we found that integrins regulated the expression of DLL4 and Jagged1, but DLL4 and Jagged1 did not regulate the expression of integrins in HUVECs expressing TR3/Nur77. We identified a novel axis of VEGF / histamine →TR3/Nur77→ integrins → DLL4 / Jagged1 in angiogenesis.

The cellular localization of TR3/Nur77 alters its cellular functions. When present in the nucleus, TR3/Nur77 functions as a transcription factor that regulates gene expression and promotes cell growth. In the cytosol, TR3/Nur77 is hyper-phosphorylated and does not have transcriptional activity, but associates with other proteins, such as PKC to inhibit PKC activity (35) or Bcl2 to promote cancer cell apoptosis (36). Our previous studies

demonstrated that the transcriptional activity of TR3/Nur77 was required for its function in angiogenesis and downregulation of the proteins in VE-cadherin associated adherent junctions and integrins (17, 29, 30). Here, we extended our findings that the transactivation domain, and the DNA-binding domain, but not the ligand-binding domain, of TR3/Nur77 were required for the regulation of DLL4 and Jagged1 induced by TR3/Nur77. Further deletion studies indicated that TR3/Nur77 regulated the expression of DLL4 and Jagged1 by various amino acid fragments. The amino acid sequence 41–60 of TR3/Nur77 was required for TR3/Nur77-regulated DLL4. The amino acid sequences 41–60, 61–80 and 101–120 were required for regulation of Jagged1 by TR3/Nur77. Amino acid sequence GRR in the DNA binding domain of TR3/Nur77 was required for the regulation of both DLL4 and Jagged1 by TR3/Nur77. Mutation of amino acid residue Serine 350 to Aspartate was unable to regulate the expression of DLL4 and Jagged1. There data extended our previous findings that TR3/Nur77 was a common target for various angiogenic factors, such as VEGF, histamine and serotonin, and regulated various angiogenic responses by targeting several signaling pathways through its various domains (15–17, 29, 30).

We also found that TR3/Nur77 played a role in sepsis induced by both CLP and LPS. Mouse survival rate significantly increased in Nur77−/− mice, indicating that expression of TR3/Nur77 was required for the development of sepsis. Our results were supported by the previous report that expression of Nur77 was decreased during the M2 phase in the gold nanoparticles attenuated sepsis in CLP mouse model (37). However, Li *et. al* reported that $Nur77^{-/-}$ mice were more susceptible to LPS-induced sepsis (38). The different results may be due to the difference in the amount of LPS used and the monitored time. The mice were injected with LPS (20 mg / kg, ip) and were monitored for 72 h in the report by Li *et. al*, while we used 13 mg / kg LPS and observed 125 h.

It is known that endothelial cells (ECs) mediate a specific and robust immune response to bacteria in sepsis through the activation of toll-like receptor (TLR) signaling, in which, DLL4 is the master regulator (review in (39). Further, DLL was activated in human lung endothelial cells and in mice treated with LPS (40). We found that DLL4 was regulated by TR3/Nur77 in sepsis. Further, we were the first to find that Jagged1 was regulated in sepsis.

Most recently, by analyzing the integrin expression profile in endothelial cells, we found that the TR3/Nur77 expression highly increased the expression of integrins α1 and β5, decreased the expression of integrins α2 and β3, but had some or no effect on the expression of integrins αv, α3, α4, α5, α6, β1 and β7 (30). Here, we found that expression of integrins were regulated by TR3/Nur77 in sepsis. Our results supported the well-known roles of integrins in sepsis.

Mouse mortality with sepsis is due to over-suppression of inflammation in the M2 phase, and TR3/Nur77 is well known as an anti-inflammatory factor, it is possible that over-suppression of inflammation was reduced in Nur77 knockout mice, resulting in the high survival rate. However, no differences in inflammatory cytokine levels or neutrophil/macrophage numbers were reported in Nur77 knockout mice in Escherichia coli-induced peritonitis (41). Our previous studies demonstrated that TR3/Nur77 plays important roles in angiogenesis to regulate tumor growth and skin wound healing (15–17, 29). It is well known

that angiogenesis plays a significant role in sepsis. In Escherichia coli-induced peritonitis, it was found that TR3/Nur77 modulated bacterial influx into the organs via increased vascular permeability by regulating both protein and mRNA expression of claudin-5, VE-cadherin, occludin, ZO-1, and β-catenin, thereby aggravating distant organ damage (41). Therefore, we will study whether TR3/Nur77 regulates sepsis by its effect on imflammatory, angiogenesis or both with tissue-specific knockout mice in the future.

Collectively, we discoveried a novel axis of VEGF / histamine → TR3/Nur77 → integrins → DLL4 / Jagged1 in angiogenesis. Various TR3/Nur77 domains were required for its regulation of DLL4 and Jagged1. Further, the survival rates of mice bearing sepsis models were increased in Nur77 knockout mice. The expressions of DLL4, Jagged 1, and integrins α1, β5, α2 and β3 were regulated in Nur77 knock mice bearing sepsis. In the future, we will further study the mechanism, by which TR3/Nur77 regulates sepsis and the expression of DLL4 and Jagged1, and develop TR3/Nur77 shRNAs into therapies for sepsis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

This work was supported by the NIH grants R01CA133235 and R03CA191463 (HZ), R01DK095873 and R21DK080970 (DZ), by the American Cancer Society grant RSG CSM 113297 (DZ), and by the Scholarships from China Scholarship Council (JP, SZ, YL, GN and CC), from Renji hospital, P.R.China (TY).

Abbreviations:

LPS	lipopolysaccharide
CLP	cecal ligation and puncture
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
EBM	Endothelial cell basal medium
HUVECs	human umbilical vein endothelial cells

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Highlights

- TR3/Nur77 regulated the expression of DLL4 and Jagged1
- DLL4 and Jagg1 mediated the cell proliferation, migration and adhesion induced by TR3/Nur77.
- DLL4 and Jagged1 differentially mediated the activation of signaling molecules induced by TR3/Nur77.
- Various amino acid fragments of TR3/Nur77 were required for its regulation of DLL4 and Jagged1.
- Deletion of TR3/Nur77 increased survival rate in mice bearing sepsis induced by CLP and LPS.
- TR3/Nur77 regulated the expression of DLL4, Jagged 1, and integrins α1, β5, α2 and β3 in sepsis.

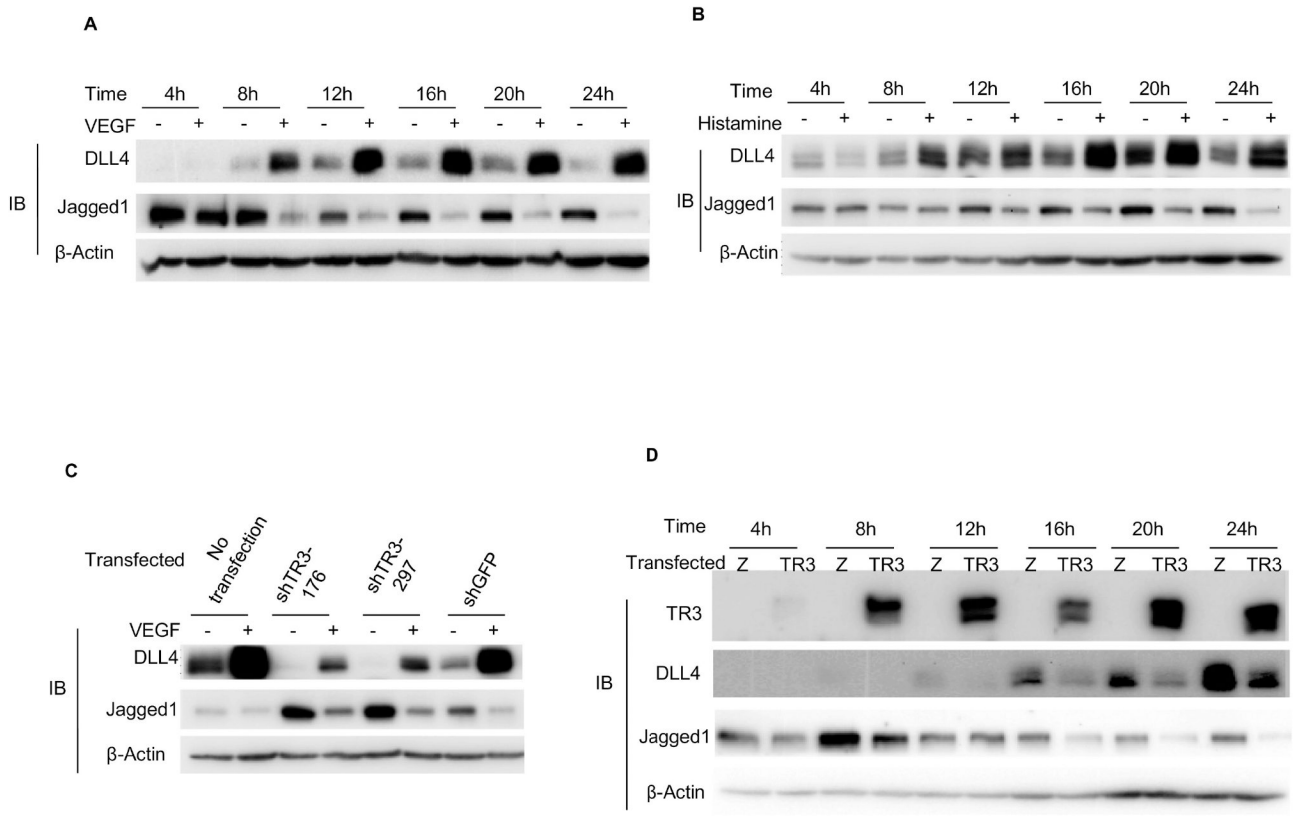

Figure 1. TR3/Nur77 mediated the expression of DLL4 and Jagged1 regulated by VEGF and histamine.

Proteins isolated from the HUVECs that were stimulated with or without VEGF (**A**) or histamine (**B**), were transduced without or with shTR3–176, shTR3–297 and shGFP as a control, and then stimulated without (−) or with (+) VEGF (**C**), were transduced with Lac Z as a control and Flag-fused TR3 for various time periods (**D**), were analyzed by immunoblotting with the antibodies against DLL4, Jagged1 and β-actin as a protein equal loading control. Data represent 3 independent experiments.

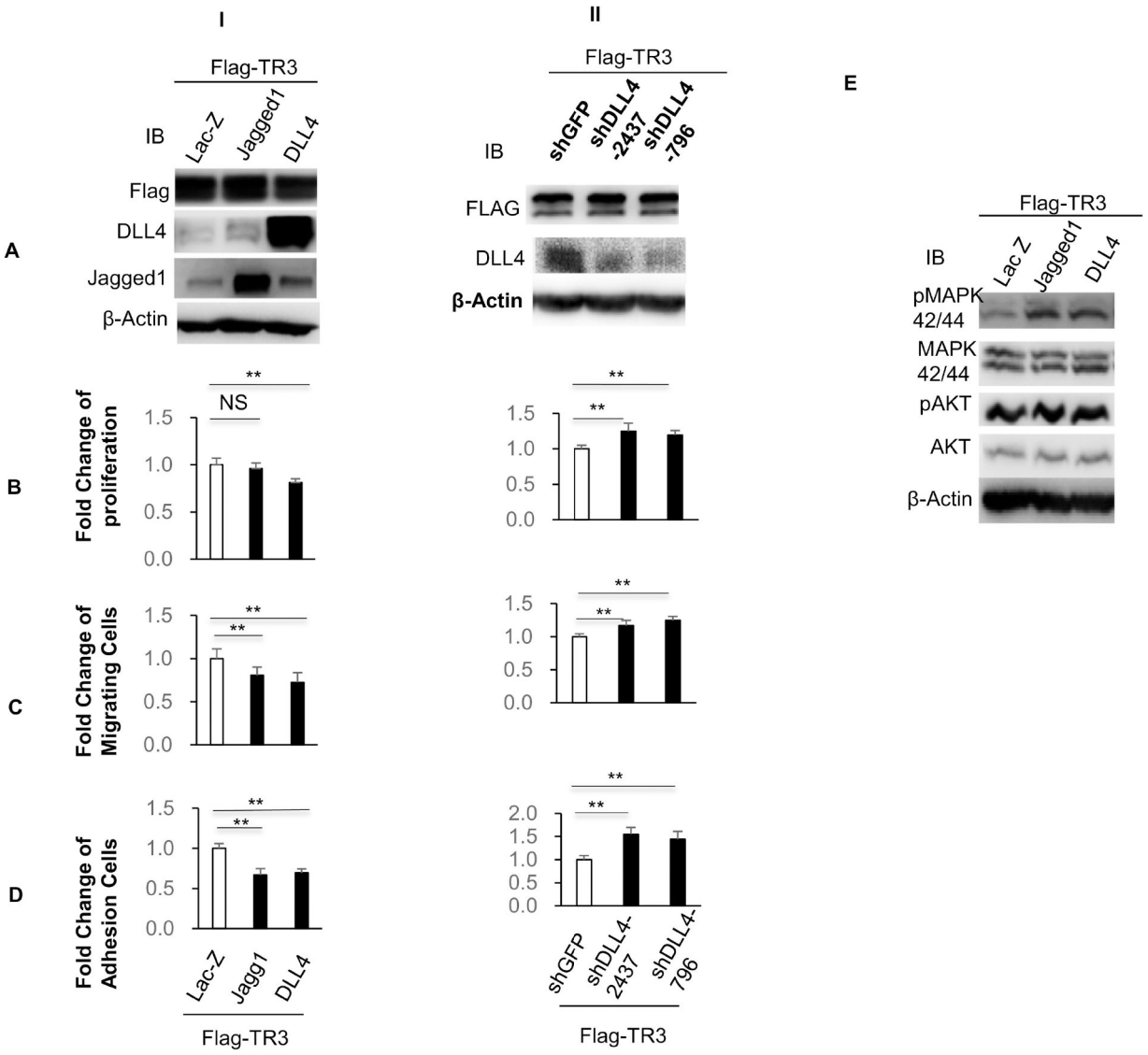

Figure 2. DLL4 and Jagged1 regulated the angiogenic responses induced by TR3/Nur77.

The HUVECs expressing Flag-TR3/Nur77 were transduced with Lac Z as a control and Jagged1 and DLL4 cDNAs (**panel I)**, shGFP as a control, shDLL4–2437 and shDLL4–796 (**Panel II**). Cellular extracts were subjected to immunoblotting analysis with the antibodies against Flag to indicate the equal expression of TR3/Nur77, DLL4, Jagged1 and β-actin as a protein equal loading control (**A**). Cells were subjected to proliferation assay (**B**), migration assay (**C**) and adhesion assay (**D**). (**E**) Cellular extracts isolated from the HUVECs expressing Flag-TR3/Nur77 and transduced with Lac Z as a control, Jagged1 and DLL4 cDNAs were subjected to immunoblotting analysis with the antibodies as indicated. Data represent 3 independent experiments (n=3, **$p < 0.01$).

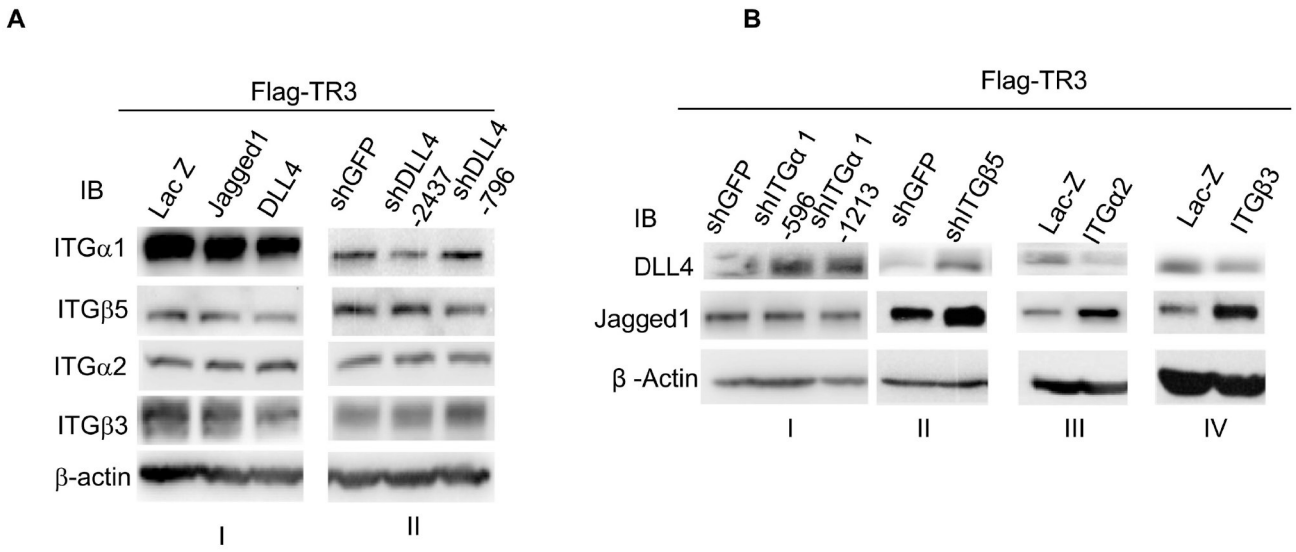

Figure 3. Integrins differentially regulated the down-regulation of DLL4 and Jagg1 induced by TR3/Nur77.

A) Cellular extracts that were isolated from the HUVECs expressing Flag-TR3/Nur77 and transduced with Lac Z as a control, and Jagged1 and DLL4 cDNAs (panel I), shGFP as a control, shDLL4–2437 and shDLL4–796 (panel II) were subjected to immunoblotting analysis with the antibodies integrins α1, α2, β3 and β5, and β-actin as a protein equal loading control. **B**) HUVECs expressing Flag-TR3/Nur77 were transduced with shGFP as a control, shITGA1–1213 and shITGA1–596 (panel I), shGFP as a control and shITGB5 (panel II), Lac Z as a control and ITGA2 (panel III), and Lac Z as a control and ITGB3 (panel IV). Cellular extracts were subjected to immunoblotting analyses with antibodies against DLL4, Jagged1 and β-actin as a protein equal loading control. Data represent 3 independent experiments.

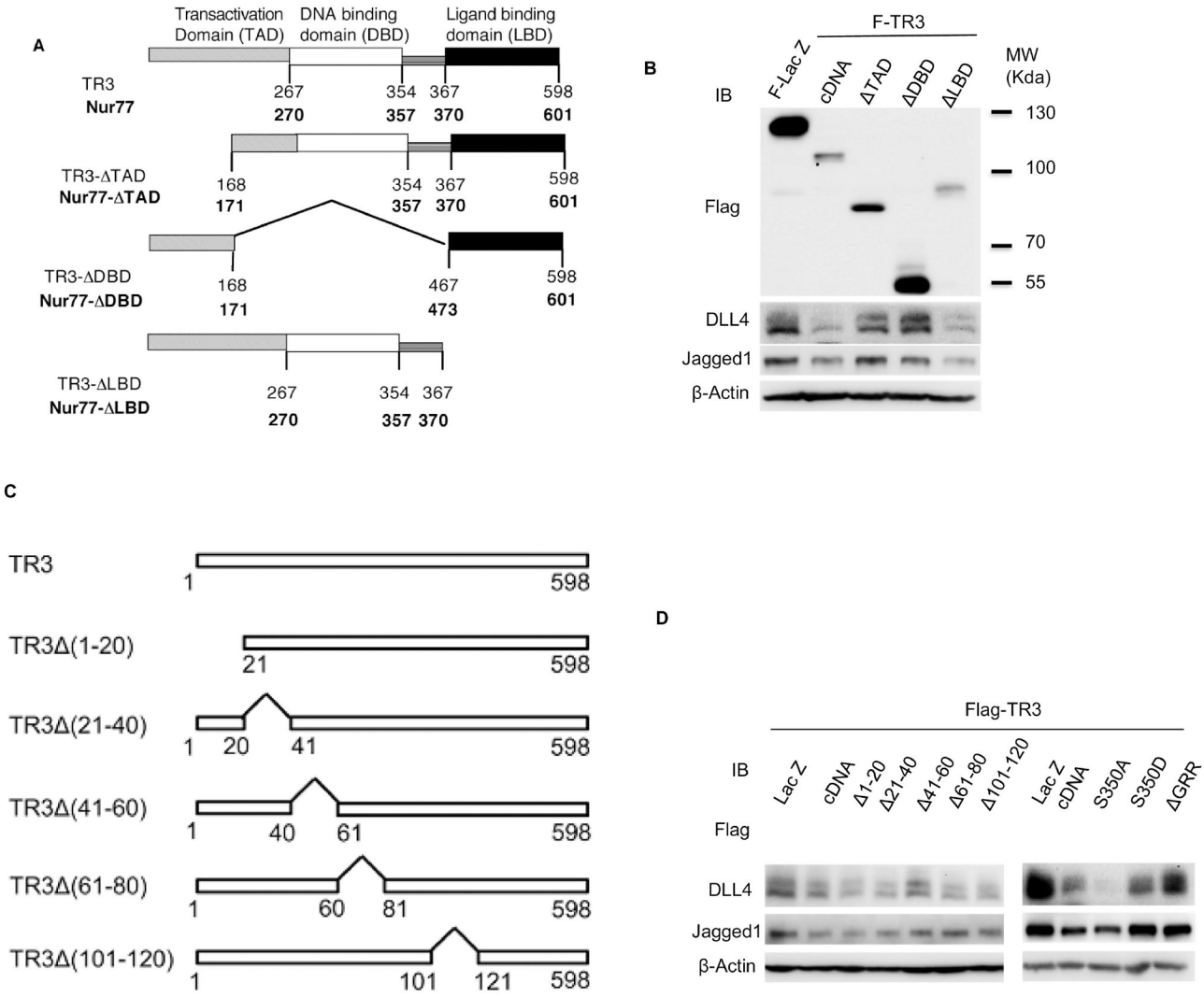

Figure 4. Various TR3/Nur77 domains were required for its regulation of DLL4 and Jagged1.

A. Schematic structure of TR3 and Nur77 genes and mutants constructed to lack TAD, DBD, or LBD domains. **B**. Cellular extracts isolated from the HUVECs that were transduced with Lac Z as a control, Flag-fused TR3, TR3ΔTAD, TR3ΔDBD, or TR3ΔLBD were subjected to immunoblotting analyses with the antibodies against Flag, DLL4, Jagged1 and β-actin as a protein equal loading control. **C**. Schematic structure of TR3 mutants in the transactivation domain. **D** Cellular extracts isolated from the HUVECs that were transduced with Lac Z as a control, Flag-fused TR3 cDNA and mutants as indicated were subjected to immunoblotting analyses with antibodies against Flag, DLL4, Jagged1, and β-actin as a protein equal loading control. Data represent 3 independent experiments.

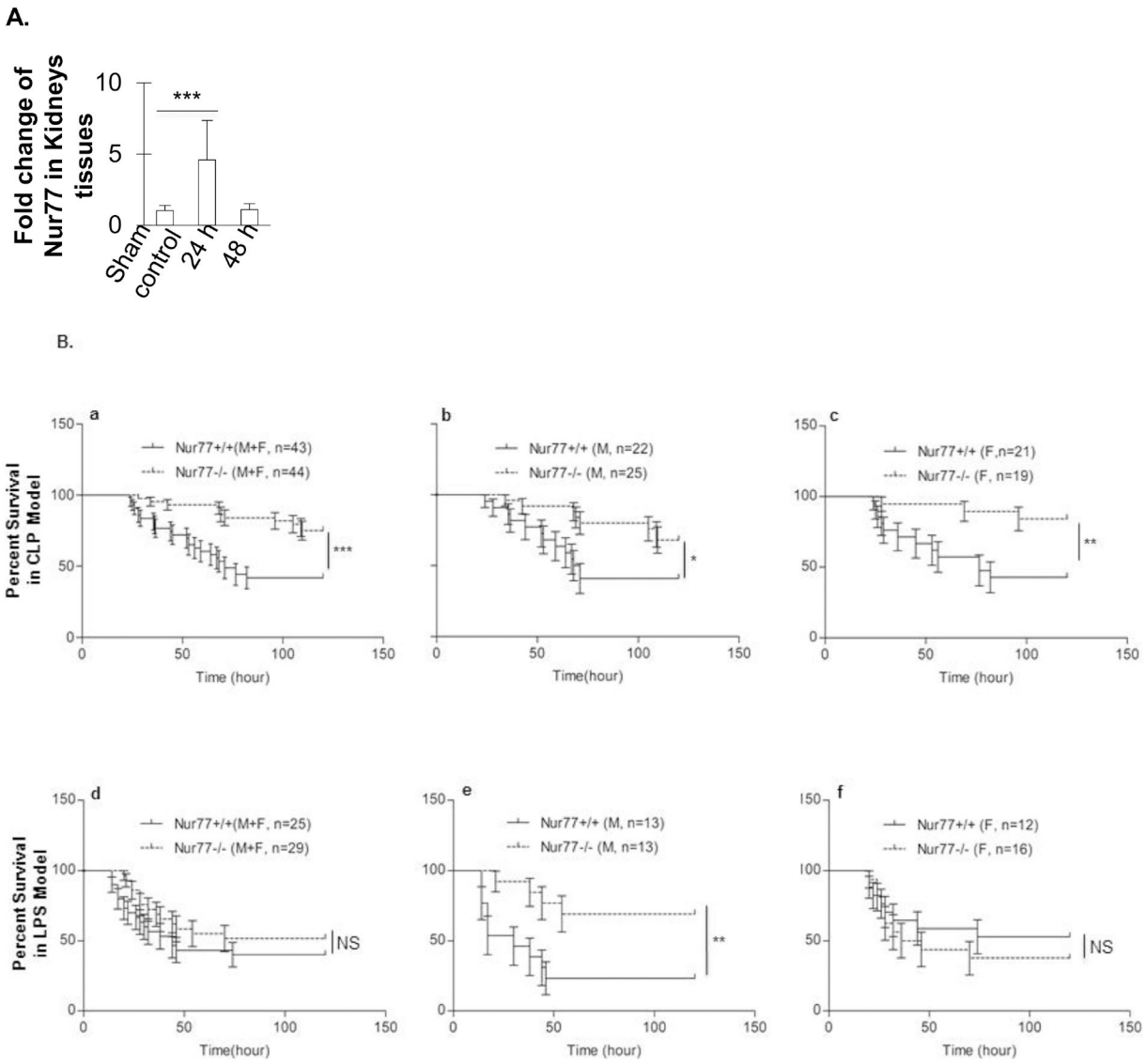

Figure 5. TR3/Nur77-induced angiogenesis played a role in sepsis.

A) RNA isolated from kidneys of mice bearing CLP-induced sepsis were subjected to RT-realtime PCR with Nur77 specific primers (n=5 mice); **B**) Mouse survival rates of Nur77−/− mice and control wildtype mice bearing CLP-induced (a-c) and LPS-induced (d-f) sepsis were analyzed with data obtained from both males and females (a and d), males only (b and e), and females only (c and f) (n as indicated, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS: no significant difference).

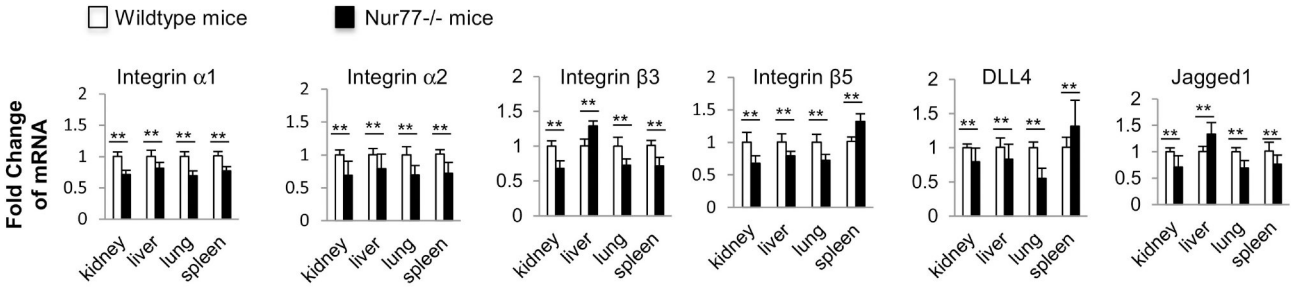

Figure 6. TR3/Nur77 regulated the expression of DLL4, Jagged 1, and integrins α1, β5, α2 and β3 in sepsis.

RNA isolated from kidneys, livers, lungs and spleens of Nur77−/− mice and control wildtype mice 24 h after CLP surgery were subjected to Realtime RT-PCR with specific primers for integrin α1, integrin α2, integrin β3, integrin β5, DLL4 and Jagged1. (n=5 mice per group, ** $p < 0.01$).